



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|---------------------------|---|----------------------------|
| re Application of:        | ) | Art Unit: 1651             |
|                           | ) |                            |
| Hidetomo KITAMURA         | ) | Examiner: L. Lankford, Jr. |
|                           | ) |                            |
| Appln. No.: 09/380,372    | ) | Washington, D.C.           |
|                           | ) |                            |
| Filed: September 1, 1999  | ) |                            |
|                           | ) |                            |
| For: NOVEL CELL LINES AND | ) | Atty. Docket: KITAMURA=1   |
| SCREENING METHODS...      | ) |                            |
|                           | ) |                            |
| Confirmation No.: 2531    | ) |                            |

DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner for Patents  
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Sir:

I, Hidetomo KITAMURA, a Japanese citizen, residing at 135, Komakado, 1-chome, Gotenba-shi, Shizuoka, 412-8513, Japan, hereby declare that I am the inventor of the above-entitled patent application, and that I received a D.V.M degree from Hokkaido University Faculty of Veterinary Medicine in March 1991.

I declare also that I have been employed by Chugai Seiyaku Kabushiki Kaisha, the assignee of this application, and have been engaged in pharmaceutical research since April 1991 and that I work as a researcher for Pharmaceutical Research Laboratory II of Chugai Seiyaku Kabushiki Kaisha.

I also declare that I have read all of the Official Actions pertaining to the above-entitled application, and am familiar with each of the references cited in the Official Actions by the Examiner.

I declare further that the following experiments were conducted by me or under my supervision and that the results and statements presented below are true and correct to the best of my knowledge.

#### **Statements**

The present invention is directed to a cell line capable of differentiating into chondrocytes and capable of differentiating into adipocytes, which cell is derived from a normal adult animal. As is indicated in Example 2 of the present specification, the cell line of the present invention is capable of differentiating into chondrocytes and capable of differentiating into adipocytes in the absence of dexamethasone. The medium used for culturing the CL-1 cells in Examples 2 and 3 of the present specification is Minimum Essential Medium a (MEMa; GibcoBRL catalogue no. 11900) containing 10% fetal calf serum (Moregate, lot no. IM1728), 100 U/ml penicillin (Banyu Pharmaceutical Co, Ltd), and 100 µg/ml streptomycin (Meiji Seika Kaisha, Ltd). Dexamethasone was absent from the culture medium used in Examples 2 and 3 of the present specification. A copy of the pertinent pages of the 1995-1996 Gibco BRL Life Technologies

Catalogue relating to MEM a medium (catalogue no. 11900) was attached to the Declaration filed March 20, 2003. It is well known that fetal calf serum and antibiotics are commonly added to medium for culturing cells.

In contrast to the presently claimed cell line, the RCJ 3.1 cells of the Grigoriadis et al. reference, J. Cell Biology, 106:2139-2151 (1988), relied upon by the examiner, were derived from mesenchymal cells of fetal rat calvaria and are unable to differentiate into adipocytes or chondrocytes in the absence of dexamethasone (abstract, left column; page 2142, right column, lines 1-11 of "Adipocyte Formation"; paragraph "Cartilage Formation" on bridging pages 2144 and 2145). Grigoriadis specifically teaches on page 2149, left column, that:

We have confirmed, however, that dexamethasone is required for progenitors to become committed to and differentiate along adipocytes and chondrocyte lineages, because RCJ 3.1 cells, subcloned in the absence of dexamethasone, only produced clones which differentiated into muscle, but not into fat or cartilage (data not shown).

Worster et al., Journal of Orthopaedic Research, 19:738-749 (2001), a copy of which was attached to the Declaration filed March 20, 2003, shows that mesenchymal progenitor stem cells obtained from adult horses (3 days to 2 years old) differentiated into chondrocytes in the absence of dexamethasone (Abstract, page 738, Material and methods, pages 740-743, and Result and Discussion, pages 744-747). In the

Materials and Methods section under Mesenchymal cell cultures (page 740, right column), it is taught that mesenchymal stem cells (MSCs) were cultured in serum-free Ham's F-12 medium containing the additives previously described (fetal bovine serum, ascorbic acid, a-ketoglutaric acid, L-glutamine, sodium penicillin, streptomycin sulfate, and HEPES buffer, as disclosed in the left column of page 740) and supplemented with TGF- $\beta$ 1. Ham's F-12 medium is a well known and commonly used nutrient culture medium in the art and its formulation is given in the pertinent pages of the 1995-1996 GibcoBRL Life Technologies Catalogue. As can be clearly seen, neither the additives supplementing Ham's F-12 medium nor the formulation of Ham's F-12 medium, the defined medium used for differentiation (chondrogenesis), contain dexamethasone. Hence, dexamethasone was not required for chondrogenesis in Worster.

The results of Example 2 of the present application and the disclosure of Worster demonstrate that the cell lines derived from a normal adult animal as recited in claim 23 of the present application can differentiate into adipocytes and chondrocytes regardless of the presence or absence of dexamethasone. By contrast, dexamethasone is required for the RCJ 3.1 cells of Grigoriadis in order to differentiate into adipocytes and chondrocytes. Accordingly, the cell line of the present invention

is clearly distinct from and patentable over the RCJ 3.1 cell line of the Grigoriadis reference.

## Experiment

### 1. Methods

Mouse CL-1 cells (accession no. FERM BP-5823) of the instant invention and the mouse ATDC5 and mouse C3H10T1/2 cells were grown to confluence on respective 24-well culture plates (5,000 cells/well, CORNING) in  $\alpha$ -MEM (GIBCO) containing 10% fetal calf serum (INTERGEN), L-ascorbic acid (50  $\mu$ g/mL, Wako pure chemicals), penicillin (100 U/mL, Meiji Seika Kaisha) and streptomycin (100  $\mu$ g/mL, Banyu), and then treated for 7 days with either vehicle control (1% ethanol) or Compound A (at 0.1, 1.0, or 10  $\mu$ mol/L). The ATDC5 cell line was obtained from mouse embryonic carcinoma (and purchased from ATCC) and has been widely used in the art as a chondrocyte precursor in studying chondrocyte differentiation. Similarly, the C3H10T1/2 cell line was obtained from mouse embryo (and purchased from ATCC) and has been widely used in the art as a multipotent precursor in studying chondrocyte, myocyte, osteoblast and adipocyte differentiation. Compound A is a chondrogenic compound disclosed in page 17 of EP1156037 A1.

The cell layers of the CL-1, ATDC5 and C3H10T1/2 cells were fixed with 4% paraformaldehyde (Wako pure chemicals) at 4°C overnight.

Cells cultured in a medium containing L-ascorbic acid can be stained with alcian blue (pH 1.0) to test whether or not stained nodules are formed. Alcian blue is a dye which is a copper phthalocyanine derivative and can stain acid polysaccharides having carboxyl groups (polyanions). It is widely used to detect acid mucopolysaccharides (glycosaminoglycan) and the distribution of sialic acid-containing glycoproteins in tissues.

After washing with distilled water, the fixed cell layers were treated with HCl (0.1 mol/L, Wako pure chemicals) for 3 minutes and stained with an alcian blue solution (pH 1.0, EM Science) overnight at room temperature. After washing with HCl (0.1 mol/L), alcian blue pigment was extracted from each of the fixed cell layers by guanidine HCl (6 mol/L, 300 µl/well, Wako pure chemicals) overnight at room temperature. For evaluating the alcian blue intensity, absorbance of the extracts at 620 nm was measured by a microplate reader (MULTISKAN).

As is apparent from Figure 3 (the effect of compound A on CL-1 and ATDC-5 cells) in the Appendix attached hereto, the alcian blue intensity significantly increased in the CL-1 cells treated with Compound A at 10 µmol/L, indicating that cartilage

formation of the CL-1 cells was significantly promoted by the treatment of Compound A. On the other hand, the treatment of ATDC5 cells with Compound A did not increase the alcian blue intensity.

Likewise, in Figure 1 of the Appendix attached hereto, which presents the effect of Compound A on alcian blue intensity of chondroprogenitor cell lines, a markedly increased alcian blue intensity was found in CL-1 cells at a concentration of 10  $\mu\text{mol/L}$ , also indicating that cartilage formation of CL-1 cells was significantly promoted by Compound A, whereas ATDC-5 cells showed a slightly decreased alcian blue intensity and C3H10T1/2 cells showed only a slightly increased alcian blue intensity.

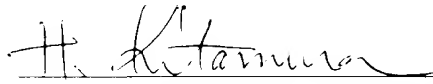
Therefore, it is concluded that Compound A promoted *in vitro* cartilage formation of the CL-1 cells of the present invention, which cells were derived from an adult animal. Compound A, however, did not promote *in vitro* cartilage formation with ATDC5 cells, which were isolated from a differentiating culture of a mouse embryonal carcinoma cell line AT805 (Atsumi et al., Cell Diff. Develop., 30: 109-116, 1990, and Takeichi et al., Develop. Biol., 87: 340-350, 1981), or with C3H10T1/2 cells, which were isolated from a mouse embryo (Catherine et al., Cancer Res. 33:3231-3238, 1973).

Figure 2 attached hereto in the Appendix shows histochemical double staining of Compound A (10  $\mu\text{mol/L}$ )-treated

chondroprogenitor cell lines with alcian blue (pH 1.0) and oil red O. Compound A is found to markedly increase chondrocyte formation in CL-1 cells (panel A is the control and panel B shows cells treated with 10  $\mu$ mol/L Compound A prior to staining). By contrast, Compound A slightly decreased alcian blue staining in ATDC-5 cells (panel D) relative to the control (panel C). Although Compound A slightly increased diffuse alcian blue intensity in C3H10T1/2 cells (panel F) relative to the control (panel E), it did not induce the formation of chondrocytes.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Dated this 29 day of October 2003

  
Hidetomo KITAMURA



# APPENDIX

Fig.1

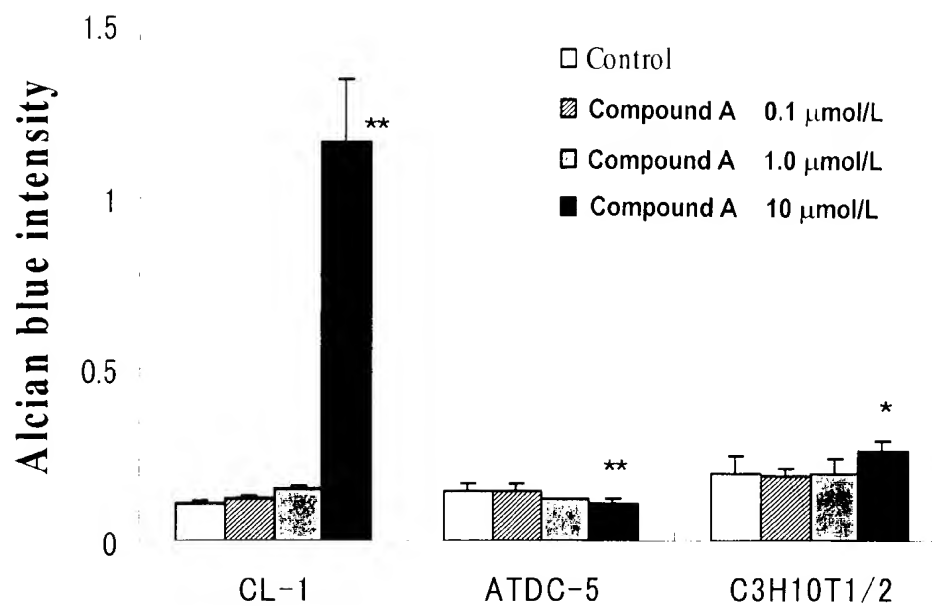


Fig.2

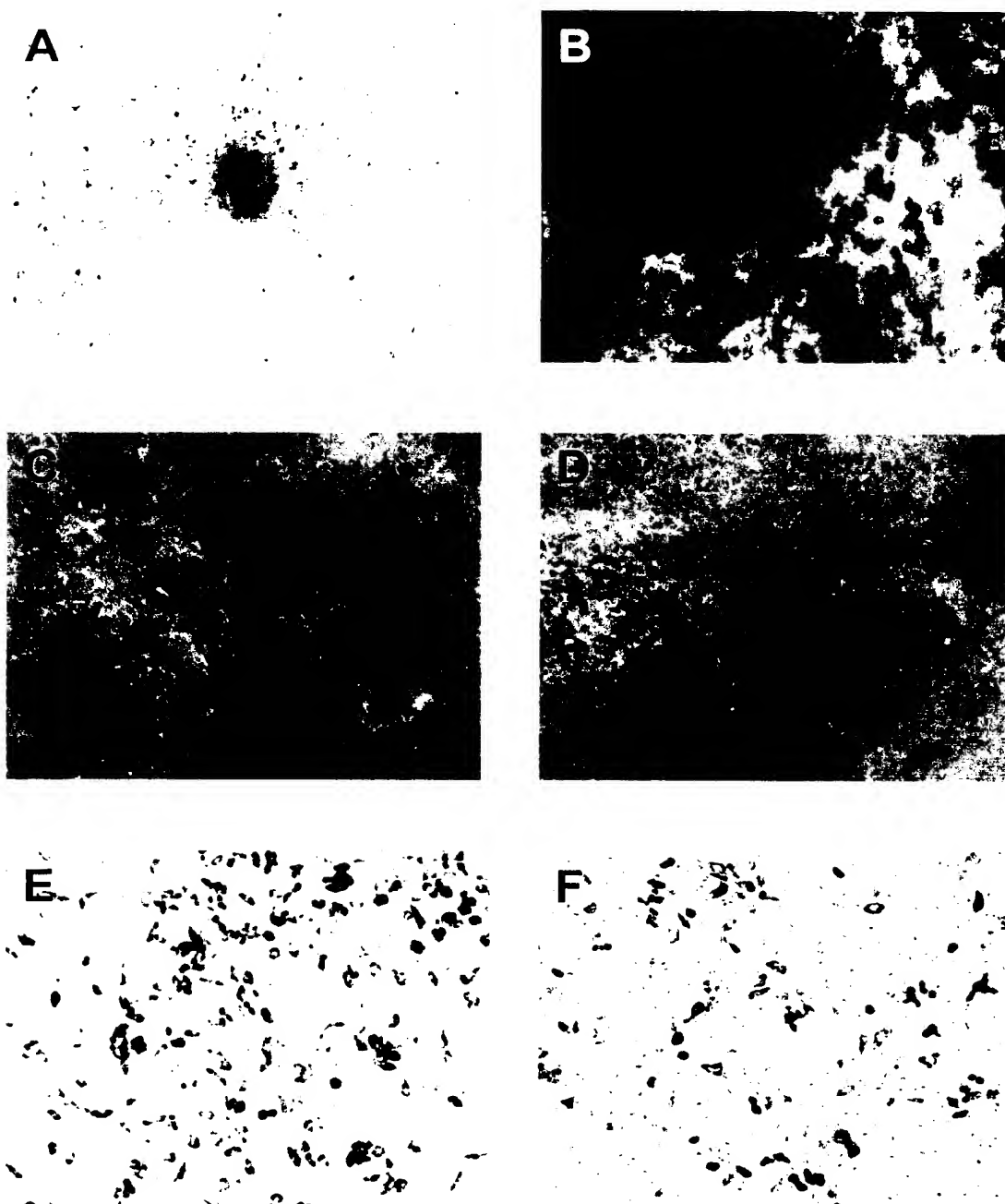
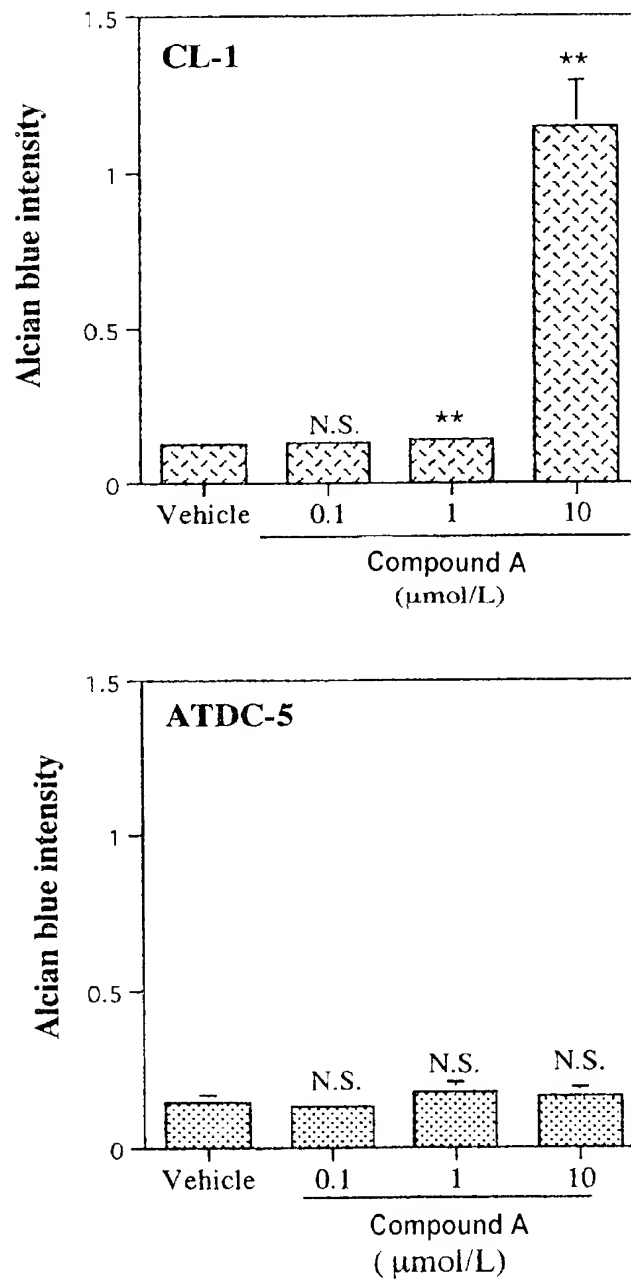


FIG. 3

Effect of Compound A on CL-1 and ATDC-5



\*\* :  $p < 0.01$  vs Vehicle (1% ethanol)

N.S.: Not Significant

Student t-test